PI:	Title	
Received: 09/15/	FOA:	Council:
Competition ID:	FOA Title:	(R01)
1 R01	Dual:	Accession Number:
IPF:	Organization:	
Former Number:	Department: Pediatrics	
IRG/SRG:	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1: 250,000 Year 2: 250,000 Year 3: 250,000 Year 4: 250,000 Year 5: 250,000	Animals: Y Humans: N Clinical Trial: N Current HS Code: HESC: N	New Investigator: N Early Stage Investigator: N
Senior/Key Personnel:	*Organization:*	*Role Category:*
MD		PD/PI
MD		Other (Specify)-Co-Investigator
		Other (Specify)-Co-Investigator
PhD		Other (Specify)-Co-Investigator
MD		Other (Specify)-Co-Investigator
MD		Other (Specify)-Other Significant Contributors

OMB Number:
Expiration Date: 06/30/

APPLICATION FOR FEDERAL ASSISTANCE (R&R)

3. DATE RECEIVED BY STATE: 09/15/

State Application Identifier:

1. * TYPE OF SUBMISSION

☐ Pre-application ☐ Application ☒ Changed/Corrected Application

2. DATE SUBMITTED: 09/15/

Applicant Identifier:

4. a. Federal Identifier:

b. Agency Routing Identifier:

5. APPLICANT INFORMATION

*** Organizational DUNS:**

* Legal Name:

Department: Pediatrics

Division: Pulmonary Biology

* Street1:

Street2:

* City:

County / Parish:

* State:

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code:

Person to be contacted on matters involving this application

Prefix: * First Name: Middle Name:

* Last Name: Suffix:

* Phone Number: Fax Number:

Email:

6. * EMPLOYER IDENTIFICATION *(EIN) or (TIN):*

7. * TYPE OF APPLICANT: M: Nonprofit with IRS Status (Other than Institution of Higher Education)

Other (Specify):

Small Business Organization Type ☐ Women Owned ☐ Socially and Economically Disadvantaged

8. * TYPE OF APPLICATION:

☒ New ☐ Resubmission

☐ Renewal ☐ Continuation ☐ Revision

If Revision, mark appropriate box(es).

☐ A. Increase Award ☐ B. Decrease Award ☐ C. Increase Duration ☐ D. Decrease Duration

☐ E. Other (specify):

* Is this application being submitted to other agencies? Yes ☐ No ☒ What other Agencies?

9. * NAME OF FEDERAL AGENCY: National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER:

TITLE: (R01)

11. * DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:

Initiation and Progression of Preterm Lung Injury with Ventilation

12. PROPOSED PROJECT:

* Start Date: 07/01/

* Ending Date: 06/30/20

*** 13. CONGRESSIONAL DISTRICT OF APPLICANT**

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: * First Name: Middle Name:

* Last Name: Suffix: MD

Position/Title: Professor of Pediatrics

* Organization Name:

Department: Pediatrics

Division: Pulmonary Biology

* Street1:

Street2:

* City:

County / Parish:

* State:

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code:

* Phone Number: Fax Number:

* Email:

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested	1,734,500.00
b. Total Non-Federal Funds	0.00
c. Total Federal & Non-Federal Funds	1,734,500.00
d. Estimated Program Income	0.00

16. * IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?

a. YES ☐ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ☒ PROGRAM IS NOT COVERED BY E.O. 12372; OR

☐ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious. or fraudulent statements or claims may subject me to criminal, civil, or administrative penalities. (U.S. Code, Title 18, Section 1001)

☒ *** I agree**

**** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.***

18. SFLLL or other Explanatory Documentation

Add Attachment | Delete Attachment | View Attachment

19. Authorized Representative

Prefix: * First Name: Middle Name:

* Last Name: Suffix:

* Position/Title: Manager

* Organization:

Department: Division:

* Street1:

Street2:

* City: County / Parish:

* State: Province:

* Country: USA: UNITED STATES * ZIP / Postal Code:

* Phone Number: Fax Number:

* Email:

*** Signature of Authorized Representative**

*** Date Signed** 09/15/

20. Pre-application Add Attachment | Delete Attachment | View Attachment

424 R&R and PHS-398 Specific
Table Of Contents

OMB Number:
Expiration Date: 08/31/20

Project/Performance Site Location(s)

Project/Performance Site Primary Location

☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name:

DUNS Number:

* Street1:

Street2:

* City: County:

* State:

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: * Project/ Performance Site Congressional District:

Project/Performance Site Location 1

☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name:

DUNS Number:

* Street1:

Street2:

* City: County:

* State:

Province:

* Country:

* ZIP / Postal Code: * Project/ Performance Site Congressional District:

Additional Location(s) Add Attachment Delete Attachment View Attachment

RESEARCH & RELATED Other Project Information

1. * Are Human Subjects Involved? ☐ Yes ☒ No

1.a If YES to Human Subjects

Is the Project Exempt from Federal regulations? ☐ Yes ☐ No

If yes, check appropriate exemption number. ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6

If no, is the IRB review Pending? ☐ Yes ☐ No

IRB Approval Date:

Human Subject Assurance Number:

2. * Are Vertebrate Animals Used? ☒ Yes ☐ No

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ☐ Yes ☒ No

IACUC Approval Date: 04/05/

Animal Welfare Assurance Number

3. * Is proprietary/privileged information included in the application? ☐ Yes ☒ No

4.a. * Does this project have an actual or potential impact on the environment? ☐ Yes ☒ No

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ☐ Yes ☐ No

4.d. If yes, please explain:

5. * Is the research performance site designated, or eligible to be designated, as a historic place? ☐ ☒

5.a. If yes, please explain:

6. * Does this project involve activities outside of the United States or partnerships with international collaborators? ☒ ☐

6.a. If yes, identify countries:

6.b. Optional Explanation:

7. * Project Summary/Abstract Add Attachment Delete Attachment View Attachment

8. * Project Narrative Add Attachment Delete Attachment View Attachment

9. Bibliography & References Cited Add Attachment Delete Attachment View Attachment

10. Facilities & Other Resources Add Attachment Delete Attachment View Attachment

11. Equipment Add Attachment Delete Attachment View Attachment

12. Other Attachments Add Attachments Delete Attachments View Attachments ☐

PROJECT SUMMARY/ABSTRACT

In the 2010 guidelines for newborn resuscitation, the International Liaison Committee on Resuscitation (ILCOR) identified gaps in knowledge, for both preterm and term infants. The gaps are the optimal maneuvers to inflate and ventilate the lungs at birth. The initiation of ventilation at birth is unique because the fetal lung must transition rapidly from fluid filled airspaces to a gas exchange, and ventilation with positive pressure at birth causes airway epithelial injury which progresses to diffuse lung inflammation. Although the lung injury is initiated with ventilation at birth, it takes time for markers of injury to develop. To overcome the confounding effects of continued ventilation, we developed a fetal sheep model of newborn resuscitation that maintains placental circulation, thus allowing us to isolate and evaluate resuscitation maneuvers designed to reduce lung injury during the transition to air breathing at birth. These maneuvers are difficult to evaluate in the clinical setting because of the necessities to resuscitate without focusing on single components of the procedure, the great variability in the clinical status of infants, and the need to continue support beyond the specific intervention. Clinicians routinely introduce new treatments, such as a sustained inflation, into newborn care without knowledge of benefits or potential for injury. The goal of this grant is to identify the safe and useful recruitment maneuvers for newborn resuscitation in preterm and near-term lambs, evaluations that cannot be easily assessed clinically. We will measure early response gene expression that activates inflammatory pathways and the location of expression of the inflammation within the lungs of preterm and term lambs. We also will test whether the severity of lung injury is dependent on gestational age (GA), and whether different acute phase injury pathways are activated during initiation of ventilation of very preterm, moderately preterm and term lungs. We will also validate protective strategies and potential therapeutic pathways in preterm newborn lambs ventilated for 4 and 24 hours. By combining a reproducible lamb model of resuscitation with advanced molecular techniques, we will determine: 1) which lung gas volume recruitment maneuvers will minimize injury, 2) how lung injury from resuscitation maneuvers differs based on the developmental stage of the lungs, and 3) if an optimum initiation of ventilation will result in decreased amplification of lung injury with continued ventilation. These innovative studies will define the molecular and physiologic responses to recruitment maneuvers in preterm and near-term lambs, resulting in new insights into how injurious pathways progress to acute and chronic lung disease. The results also will allow us to identify potential treatment targets and biomarkers for the field. These studies will provide a scientific basis for ILCOR recommendations for clinical practices that are very difficult to verify by clinical trials.

Although approximately 10% of all newborns and the majority of very low birth weight preterm infants need some assistance to breathe at birth, the optimal maneuvers to inflate and ventilate the lungs have not been defined. It is easy to injure the preterm lung at birth and mechanical ventilation contributes to the long term disability in very preterm infants. These studies will determine beneficial lung recruitment maneuvers for both term and preterm infants, and advance our knowledge on inflammatory pathways activated by ventilation at birth.

FACILITIES and RESOURCES –

Laboratory: The biochemistry, molecular biology, cell biology, protein analysis including immuno histology and morphological analyses will be performed in Drs. lab at the

laboratories of Division of Pulmonary Biology, including laboratory that studies airway injury in transgenic mice. The laboratory culture is for free access to reagents and techniques and open scientific discussion, which greatly enhances the quality of work proposed. The lab is fully equipped to run standard molecular biology assays, FACS analysis, protein assays and have high speed centrifuges, PCR machines, waterbaths, -20°C freezers, -70°C freezer, fume hood and radiation work area. The lab is next to the Pulmonary Biology core morphology and cell culture facilities to we have complete access. The laboratory space and work environment is conducive to the proposed experiments.

Animal: The animal work will be performed in . There also are facilities at for pilot experiments and proof of concept studies. Any based animal work will be performed in approved animal care facilities of . There is also a 1000 sq. ft dedicated space where preterm lamb ventilation, physiologic measurements and surgeries can be performed.

Computer: All key personnel have Macintosh networked computers in the offices connected to the and the . The department of Information Services at is available for consultation. All the laboratories have computers and workstations with appropriate software for FACS analysis graphics morphology and morphometry.

Office: Dr. has a 120 sq ft. independent office on the adjacent to the laboratory land adjacent to the offices, as well as other faculty members of the Division of Pulmonary Biology and Division of Immunology.

Clinical: Although no clinical work is proposed here, Dr. office is adjacent to offices of several clinical and research faculty of the division of Neonatology. This allows informal discussions and enhances interactions leading to better clinical relevance of the experimental design.

Other: Work involved in this project will be performed in core laboratories that have expertise in gene expression analysis and information, morphology and cell culture core. This improves efficiency of work.

FACILITIES and RESOURCES –

Laboratory - The facilities include an AQIS approved large sheep shed on the for animal work not involving recovery surgery. The large animal facility on the campus has dedicated animal space and adjacent laboratory facilities and includes surgical theatre suitable for the proposed studies. The research station has ample pasture for feeding pregnant ewes. We will have unencumbered access to the facility and have used these facilities for the past several years. The surgical necropsy and immediate tissue processing as well as surgeries are performed here.

Animal: Sheep for the project will be managed by the . Ewes in good condition are date-mated according to our requirements, scanned by ultra-sound to confirm singleton pregnancies and transported to the research station.

Computer: All computer facilities and onsite support required for this project are available within the offices of the

Office: Dr. has independent office space in the as the head of division of Obstetrics and Gynecology. Dr. is the Director of the Large Animal Facility and has his office, a separate fully equipped laboratory and computers, there. His lab is a P2 facility with complete tissue culture and processing capabilities. Office space within the premises of School of Women's and Infants' Health. Perinatal research laboratories are available to Dr. and other traveling faculty investigators for this project.

Clinical: N/A

Other:

EQUIPMENT

-The core facilities of the Division of Pulmonary Biology are housed on the adjacent to the laboratory space and are available to all investigators. We will use the core morphology and core cell culture facilities , both of which are fully equipped. Shared equipment includes 3 flow cytometry machines including flow sorting abilities, a confocal microscope, light and fluorescent microscopes equipped for morphometry, a laser capture facility, equipment for measuring and imaging RNA and DNA, fluorescent and non-fluorescent plate readers, ELISA reader, X-ray film processor, low speed centrifuge, tissue culture facility, liquid nitrogen cryostorage system, a cold room with walk-in refrigerator and -20°C freezer rooms, vacuum oven, gel dryer system, incubator shakers, a cytocentrifuge, ultracentrifuges, autoclave, liquid scintillation counter and UV crosslinker. Molecular Dynamics Phosphorimaging system and computer workstations are available as shared facilities housed on the . In add ition a fully equipped lamb intensive care unit including a portable ultrasound machine is housed on the of the research space. The veterinary services in have sheep housing and a large animal care facility. All the equipment needed for the proposed experiments are already present in .

– The Research station at the has equipment for ultrasound machine, centrifuges, surgical theatre, freezers, mechanical ventilators, resuscitation beds, microscopes, blood gas machine and all other associated necessary equipment needed for fetal surgery, fetal ventilation, delivering ewes and tissue processing. The wet laboratory space has a flow cytometer, fluorescent and non-fluorescent plate readers, centrifuges, refrigerator, freezer, autoclave and other standard laboratory equipment.

OMB Number:
Expiration Date: 06/30/20

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: * First Name: Middle Name:
* Last Name: Suffix: MD
Position/Title: Professor of Pediatrics Department: Pediatrics
Organization Name: Division: Pulmonary Biology
* Street1:
Street2:
* City: County/ Parish:
* State: Province:
* Country: USA: UNITED STATES * Zip / Postal Code:
* Phone Number: Fax Number:
* E-Mail:

Credential, e.g., agency login:

*** Project Role:** PD/PI **Other Project Role Category:**

Degree Type: MD
Degree Year:

***Attach Biographical Sketch** Add Attachment Delete Attachment View Attachment
Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

PROFILE - Senior/Key Person 1

Prefix: * First Name: Middle Name:
* Last Name: Suffix: MD
Position/Title: Assistant Professor Department: Pediatrics
Organization Name: Division: Pulmonary Biology
* Street1:
Street2:
* City: County/ Parish:
* State: Province:
* Country: USA: UNITED STATES * Zip / Postal Code:
* Phone Number: Fax Number:
* E-Mail:

Credential, e.g., agency login:

*** Project Role:** Other (Specify) **Other Project Role Category:** Co-Investigator

Degree Type: MD
Degree Year: 2001

***Attach Biographical Sketch** Add Attachment Delete Attachment View Attachment
Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Senior/Key Person 2

Prefix: * First Name: Middle Name:
* Last Name: Suffix:
Position/Title: Associate Professor Department: Pediatrics
Organization Name: Division: Pulmonary Biology
* Street1:
Street2:
* City: County/ Parish:
* State: Province:
* Country: USA: UNITED STATES * Zip / Postal Code:
* Phone Number: Fax Number:
* E-Mail:

Credential, e.g., agency login:

*** Project Role:** Other (Specify) **Other Project Role Category:** Co-Investigator

Degree Type: MD
Degree Year: 1986

***Attach Biographical Sketch** Add Attachment Delete Attachment View Attachment
Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

PROFILE - Senior/Key Person 3

Prefix: * First Name: Middle Name:
* Last Name: Suffix: PhD
Position/Title: Research Assistant Professor Department: Obstetrics and Gynecology
Organization Name: Division:
* Street1:
Street2:
* City: County/ Parish:
* State: Province:
* Country: * Zip / Postal Code:
* Phone Number: Fax Number:
* E-Mail:

Credential, e.g., agency login:

*** Project Role:** Other (Specify) **Other Project Role Category:** Co-Investigator

Degree Type: PhD
Degree Year: 2006

***Attach Biographical Sketch** Add Attachment Delete Attachment View Attachment
Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Senior/Key Person 4

Prefix:
* First Name:
Middle Name:
* Last Name:
Suffix: MD
Position/Title: Professor
Department: Obstetrics and Gynecology
Organization Name:
Division:
* Street1:
Street2:
* City:
County/ Parish:
* State:
Province:
* Country:
* Zip / Postal Code:
* Phone Number:
Fax Number:
* E-Mail:

Credential, e.g., agency login:

*** Project Role:** Other (Specify)
Other Project Role Category: Co-Investigator

Degree Type: MD
Degree Year: 1986

***Attach Biographical Sketch** Add Attachment Delete Attachment View Attachment
Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

PROFILE - Senior/Key Person 5

Prefix:
* First Name:
Middle Name:
* Last Name:
Suffix: MD
Position/Title: Professor
Department: Pediatrics
Organization Name:
Division: Pulmonary Biology
* Street1:
Street2:
* City:
County/ Parish:
* State:
Province:
* Country: USA: UNITED STATES
* Zip / Postal Code:
* Phone Number:
Fax Number:
* E-Mail:

Credential, e.g., agency login:

*** Project Role:** Other (Specify)
Other Project Role Category: Other Significant Contributors

Degree Type: MD
Degree Year: 1973

***Attach Biographical Sketch** Add Attachment Delete Attachment View Attachment
Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
, MD, PhD	Professor of Pediatrics
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
	B.A.		Biology
	M.D.		Medicine
	Ph.D.		Cell Biology

A. Personal Statement

I have had consistent funding from NIH to explore lung maturation and disease in the preterm. This work has evolved from an initial focus on surfactant function and metabolism to modulators of lung maturation. I extensively studied the effects of antenatal corticosteroids and thyroid axis hormones on the developing lung. More recently the focus has been on the lung inflammation pathway to maturation and injury of the preterm. I have a 20 year collaboration with colleagues in to develop models of fetal inflammation that include intra-amniotic LPS and live Ureaplasma to model chorioamnionitis in fetal sheep. The focus on the fetal lung has expanded to studies of the fetal inflammatory response syndrome and modulation of innate immunity in the fetus. As part of the interest in fetal/neonatal responses to inflammation, we are exploring lung injury resulting from neonatal resuscitation/ventilation practices. As clinical correlates, we presently are evaluating potential effects of fetal exposures to infection in late-preterm infants. I also am the PI for the site for a 5-site project to identify biomarkers of BPD. This research proposal is a direct collaboration between me and , MD, who is in the third year of 5 years of support from . This work on airway injury and repair is directly relevant to our clinical projects on lung outcomes of late preterm infants and to identify biomarkers and outcomes of very preterm infants.

B. Positions and Employment

Other Experience and Professional Memberships

Honors

C. Selected publications (from 313 peer-reviewed publications and 177 Chapters and Reviews)

1.
2.
3.
4.
5.
6.
7.
8.
9.
10. ,
11.
12.
13.
14.
15.

D. Ongoing Research Support

02/03/20 -02/04/20 Mechanisms of Fetal Inflammatory Response Syndrome Induced by Chorioamnionitis

This project uses a chorioamnionitis model in fetal sheep to explore the mechanisms and modulators of the multi-organ fetal response to inflammation. The timing and progression of fetal responses in lungs, liver, immune e response organs, and the brain will be evaluated.

R01 – 09/01/ -06/30/

NIH-NHLBI
Late Preterm Birth, Ureaplasma Species, and Childhood Lung Disease
This study includes a human research component to culture the chorioamnion and assess histopathology for later preterm deliveries. Ureaplasma that are isolated will be extensively characterized for the MBA virulence factors. The fetal responses to in utero exposures will be evaluated using white blood cells recovered from cord blood. Children exposed to Ureaplasma will have lung function assessments. In parallel studies, fetal sheep will be studied for the variability of the MBA as it relates to virulence and the innate fetal responses to the organisms.

U10- 05/01/ -04/30/
NIH-NHLBI
Prematurity and Respiratory Outcomes Program. This is a cooperative 5-center program to identify biomarkers for BPD, phenotype the lung disease in VLBW infants, and assess lung function at 1 yr. We will perform characterization of innate immunity using lymphocytes, glycan genotype and phenotype, and evaluations of SP-A and SP-D.

NHMRC– Chief Investigator 06/01/ -05/31/ Effective Treatment of Ureaplasma to Prevent Preterm Birth. These studies explore how antibiotics can be used to treat women with intra-amniotic colonization with Ureaplasma parvum. The studies are performed with sheep given intra-amniotic Ureaplasma. All studies conducted in .

- Consultant
Improving Outcomes for Infants 1.5-2.5kg 12/20 - Ongoing
This project is to perform a pilot project using a randomized cluster design to train local caretakers and families of newborns to care for their infants using strategies to supplement WHO guidelines. The project is in the disadvantaged northern provinces of .

Completed Research In Last 3 Years

PI 04/01/ -03/31/
NIH-NICHD
Neonatal Resuscitation and Preterm Lung Injury
This project is to determine factors contributing to the injury response of the preterm lung to the initiation of ventilation using continuous positive airway pressure, oxygen, and mechanical ventilation. The studies are translational explorations of which components of neonatal resuscitation injure the preterm lung and how that injury is signaled.

R01 09/01/ -08/31/
NIH/NHLBI
New Mediators of Clinical Lung Maturation
The studies explore the signaling resulting in inflammation via toll-like receptors and how the inflammation induces lung maturation in fetal sheep. The studies are performed as a consortium with colleagues in . We found that inflammatory cells recruited to the fetal lungs are essential for a lung maturation and IL-1 is an essential early signaling cytokine for induced lung maturation.

R21 -PI 08/01/ -07/31/
NIH-NIAID
Postnatal Consequences of Fetal Inflammation
This project with collaborators in Western explored the hypothesis that antenatal exposure to inflammation will alter postnatal immune responses and airway reactivity in sheep born spontaneously at term. We evaluated lung responses in newborn and 8wk old lambs following intrauterine exposures to LPS and Ureaplasma parvum.

NHMRC - 06/01/ -12/31/
This project explored the pathogenicity of different serovars of Ureaplasma parvum given at different doses by intrauterine injection early in gestation in sheep. One specific aim is to treat colonized sheep with antibiotics to test ability to clear organisms. The goal is to learn how Ureaplasma colonizes of the fetus as a model of the chronic chorioamnionitis that is common in women.

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
	Assistant Professor
eRA COMMONS USER NAME (credential, e.g., agency login)	

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
	B.A.	1992-1996	Religion
	M.D.	1997-2001	Medicine

A. Personal Statement

I am interested in ventilator-induced lung injury and the progression of inflammation and injury toward bronchopulmonary dysplasia (BPD) in preterm infants. The focus of my current work is to evaluate the biochemical processes and signaling that trigger the pro-inflammatory response seen with initiation of ventilation at birth and the repair of airways after this injury. I have been working with a sheep model of resuscitation injury in collaboration with our colleagues at for the past 8 years. Our research group has 21 years of collaboration with the on both a model of chorioamnionitis and ventilation-induced injury in preterm sheep. My current K08 funding supports my further training in the fields of lung injury and immunology.

B. Positions and Honors.

Positions and Employment

Honors

C. Selected peer-reviewed publications (in chronological order).

1.

2.

3.

4.

5.

6.

7.

8.

9.

10.

11.

12.

13.

14.

15.

16.

17.

18.

19.

D. Research Support

<u>Ongoing Research Support</u>

K08- 08/01/ - 7/30/
NIH-NHLBI
Lung Injury with Resuscitation of the Preterm

The major goal of this project is to study the effects of initiating ventilation in the preterm lamb and means of modulating the inflammatory cascade. The educational components provide additional graduate work needed to enable the PI to become an independent investigator.

<u>Completed Research Support</u>

R01 4/1/ – 3/31/
NIH/NICHD
Neonatal Resuscitation and Preterm Lung Injury
This project is to determine factors contributing to the injury response of the preterm lung to the initiation of ventilation using continuous positive pressure, oxygen, and mechanical ventilation. The studies are translational explorations of which components of neonatal resuscitation injure the preterm lung and how.
Role: Collaborator

NIH 7/1/ - 7/1/
NIH/NICHD

Procter Scholar 7/1/ – 7/1/

NIH Loan Repayment Program 7/1/ – 7/1/ , 7/1/ -7/1/
NIH/NHLBI
Role: Recipient

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
	.	1983	Medicine
		1986	Pediatrics
Born		1986	Pediatrics
		1990	Pediatrics
		1993	
i		1995	

A. Personal Statement

My research interests, publication profile and NIH funding are focused on understanding the pathogenesis and mechanisms of fetal lung inflammatory responses. My laboratory generated sheep specific reagents to enable studies of lung inflammation including cloning of several sheep cytokines and growth factors. The initial experimental sheep model was chorioamnionitis induced by LPS. Thereafter, along with Drs. , we investigated lung inflammatory responses after different styles of mechanical ventilation. The experiments then evolved to understanding the pathogenesis of fetal inflammation chorioamnionitis induced by injection of live Ureaplasma in sheep –funded by a R-01 grant (PI –). The target human disease for all these models is BPD, and using these models, our laboratory is focused on understanding the interactions between antenatal and postnatal inflammation leading to BPD. Since BPD in humans occurs exclusively in the preterm whose lung and the immune system are developing, the preterm sheep models developed by our group, are particularly relevant. These sheep studies are performed in collaboration with Dr. . Using these models, I have also been able to make important contributions to the understanding of innate immunity in the developing fetus. Dr. and I are mentors for Dr. for his K-08 award from the to study the pathogenesis and mechanisms of ventilator induced lung injury in the preterm. Drs. travel to every year to work on the sheep studies. These interactions demonstrate a close-knit collaborative relationship between the co-investigators, in which my forte is molecular studies of lung inflammation and developmental immunology. Our studies in the pathogenesis of lung injury in the preterms also involve human studies funded by a R-01 grant (– PI) and my participation as an alternate PI of the NICHD funded Neonatal Network Center Grant. Therefore, the investigating team for the present grant has a wider appreciation and expertise of potentially translating basic observations from the sheep model to bedside clinical applications for evidence based resuscitation guidelines.

B. Positions and Honors.

POSITION AND EMPLOYMENT

HONORS/AWARDS:

CERTIFICATIONS:

PROFESSIONAL MEMBERSHIPS:

American Academy of Pediatrics (since)
Society for Pediatric Research (since
American Physiological Society (since)

C. Selected publications (Out of 52 peer-reviewed, 21 invited reviews)

1.

2.

3.

4.

5.

6.

7.

8.

9.

10.

11.

12.

13.

14.

15.

D. Research Support
Ongoing Research Support

NIH-NHLBI R01

NIH-NICHD R01

NIH U10-HD

MERCK (MSG):

PROJECTS COMPLETED IN THE PAST THREE YEARS

NIH/NHLBI R01-

NIH-AI R21-

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
	BMLSc		Biomedical Science
	(HONS)		Biomedical Science
	PhD		Medical Research
		2010	Medical Education Research
	-	2006 - 2009	Postdoctoral Training (Medical Research)
		-	

A. Personal Statement

I am a Research Assistant Professor at . My primary research goal is the application of translational research to further our understanding and treatment of preterm birth and its associated sequaele. My research in this field presently focuses on understanding the role(s) played by maternal and fetal tissues in the development of *in utero* inflammation and fetal injury especially that subsequent to intra-uterine *Ureaplasma* infection.

I trained in biomedical science at The and pursued postgraduate study in medical research, completing a PhD at The . My postdoctoral training in medical research included a three year Fellowship at The ( ) before returning to to take up my current position.

As an early career researcher I am presently developing a novel area of fetal biology – the role played by the fetal skin in the development of inflammation and preterm birth. I am undertaking this work as part of a major research collaboration headed by Professors . This collaboration utilizes the sheep model of *in utero* infection and inflammation as a basis for the investigation of novel methods of preventing and treating preterm birth. These studies are now in their 21st consecutive year and have resulted in world-wide changes in clinical practice.

In addition to my own research, I am responsible for overseeing the scientific direction of our sheep research facilities. These facilities directly support the studies outlined in this application. All necessary facilities are currently fully resourced and operational. Development and construction of most of these facilities was initiated by Professors . These resources include sheep breeding facilities at ; low intensity animal research facilities in high intensity surgical and management facilities within the Large Animal Facility on the University campus; and general laboratory facilities within our hospital and university.

B. Positions
Positions and Employment

Other Experience and Professional Memberships

Honours

2003:

2003:
2004:

C. Selected peer-reviewed publications (in chronological order)

1)

2)

3)

4)

5)

6)

7)

.

8)

9)

10)

11)

12)

13)

14)

15)

D. Research Support

2010:

2010:

2010:

2010:

2009:

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	Professor of Obstetrics and Gynecology
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
		1976	Medicine
		1984	ObGyn
		1986	Medical research
		1992	Maternal Fetal Medicine
		1996	ObGyn

A. Personal Statement

I am a Maternal-Fetal Medicine Specialist

which work together for the common goal of discovering ways to improve the health of women and babies. I have a career-long research commitment to the prevention of preterm birth and my research in this field now focuses on long-standing intra-uterine infection, in particular with *Ureaplasma*.

I trained in medicine at T and pursued postgraduate training in obstetrics in My postgraduate training in Maternal Fetal Medicine included a two year Fellowship at

The sheep research facilities available in for conduct of the studies outlined in this application are fully resourced and operational. Development and construction of most of these facilities have been initiated by Professor and myself. These resources include sheep breeding facilities at low intensity animal research facilities in high intensity surgical and management facilities within the Large Animal Facility on the University campus; and general laboratory facilities within our Hospital and University.

I am also involved in studying the life-long implications of events before and soon after birth. In I was initiator and principal investigator of a major cohort study of 2900 children followed from 16 weeks gestation to adulthood, designed to investigate the developmental origins of disease. This Study, known as the is one of the largest and most complete of its type in the world. The children in this study are now 20-22 years of age and remain under follow-up with retention of at least 70%.

Career Summary (July):
Number of publications: 228
Number of citations: 4064
Average citations per item: 17.82

H index: 35

B. Positions and Honors
Positions and Employment

Other Experience and Professional Memberships

Honors

C. Selected peer-reviewed publications (in chronological order)
(publications selected from 228 peer-reviewed original manuscripts)
1.

2.

3.

4.
s
5.

6.

7.

8.

9.

10.

11.

12.

13.

14.

15.

D. Research Support

National Institutes of Health (NIH)

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
M.D.	Professor of Pediatrics
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
	B.A.	1969	Chemistry
	M.D.	1973	Medicine
		1973-1976	Intership/Residency
		1976-1978	

A. Personal Statement

I am a senior investigator with an active and long-standing interest in lung formation, function, and repair.

I have had long interest in the genetic pathways critical for epithelial cell differentiation in the lung and for many years have studied the transcription factors and signaling molecules critical for lung formation, function, and repair, including Sox KLF-5 and Ets family (SPDEF) proteins. My laboratory has extensive experience in histology, immunohistochemistry, the production and analysis of complex transgenic mice with endodermal phenotypes.

B. Positions and Honors:

2009
2003
1995
1994
1988
1985

.

Honors:

C. Selected peer-reviewed publications. (Selected from 450 peer-reviewed manuscripts)

<u>Most relevant to the current application</u>

1.

2.

3.

4.

5.

<u>Additional publications of important to the field (in chronological order)</u>

1.

2.

3.

4.

5.

6.

7.

8.

9.

10.

D. Research Support

Role: PI

Role: PI

Role: PI

Role: PI

Completed Research Support

PHS 398 Cover Page Supplement

OMB Number:

1. Project Director / Principal Investigator (PD/PI)

Prefix:

* First Name:

Middle Name:

* Last Name:

Suffix: MD

2. Human Subjects

Clinical Trial? ☒ No ☐ Yes

* Agency-Defined Phase III Clinical Trial? ☐ No ☐ Yes

3. Applicant Organization Contact

Person to be contacted on matters involving this application

Prefix:

* First Name:

Middle Name:

* Last Name:

Suffix:

* Phone Number:

Fax Number:

Email:

* Title: Manager

* Street1:

Street2:

* City:

County/Parish:

* State:

Province:

* Country: USA: UNITED STATES

* Zip / Postal Code:

PHS 398 Cover Page Supplement

4. Human Embryonic Stem Cells

* Does the proposed project involve human embryonic stem cells? ☒ No ☐ Yes

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://stemcells.nih.gov/research/registry/. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

Cell Line(s): ☐ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

PHS 398 Modular Budget, Periods 1 and 2

OMB Number:

Budget Period: 1

Start Date: 07/01/ End Date: 06/30/

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	
Consortium F&A	
* Total Direct Costs	

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.		53		
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number) Department of Health and Human Services,

Indirect Cost Rate Agreement Date 07/02/

Total Indirect Costs

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($)

Budget Period: 2

Start Date: 07/01/ End Date: 06/30/

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	
Consortium F&A	
* Total Direct Costs	

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	MTDC	53		
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number) Department of Health and Human Services,

Indirect Cost Rate Agreement Date 07/02/

Total Indirect Costs

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($)

PHS 398 Modular Budget, Periods 3 and 4

Budget Period: 3

Start Date: 07/01/ End Date: 06/30/

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	
Consortium F&A	
* Total Direct Costs	

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
		53		
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): Department of Health and Human Services,

Indirect Cost Rate Agreement Date: 07/02/

Total Indirect Costs:

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($):

Budget Period: 4

Start Date: 07/01/ End Date: 06/30/

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	
Consortium F&A	
* Total Direct Costs	

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.		53		
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): Department of Health and Human Services,

Indirect Cost Rate Agreement Date: 07/02/

Total Indirect Costs:

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($):

PHS 398 Modular Budget, Periods 5 and Cumulative

Budget Period: 5

Start Date: 07/01/ End Date: 06/30/

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	
Consortium F&A	
* Total Direct Costs	

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1		53		
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): Department of Health and Human Services,

Indirect Cost Rate Agreement Date: 07/02/

Total Indirect Costs:

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($):

Cumulative Budget Information

1. Total Costs, Entire Project Period

*Section A, Total Direct Cost less Consortium F&A for Entire Project Period	$
Section A, Total Consortium F&A for Entire Project Period	$
*Section A, Total Direct Costs for Entire Project Period	$
*Section B, Total Indirect Costs for Entire Project Period	$
*Section C, Total Direct and Indirect Costs (A+B) for Entire Project Period	$

2. Budget Justifications

Personnel Justification		Add Attachment	Delete Attachment	View Attachment
Consortium Justification		Add Attachment	Delete Attachment	View Attachment
Additional Narrative Justification		Add Attachment	Delete Attachment	View Attachment

MODULAR BUDGET PERSONNEL JUSTIFICATION

Senior/Key Persons

, MD, PhD (1.8 calendar months for years 1 to 3 and 1.2 calendar months for years 4 to 5) is the Principal Investigator for this grant. This grant was developed by Drs. and all aspects of the work will be supervised or performed by them. The sheep work in will be done with both Drs. in attendance. Dr. assists with surgery and with all autopsies. The laboratory work will be completed in along with data analysis and manuscript writing either directly by or supervised by Dr.

, MD (2.4 calendar months) is a critical co-investigator as he is presently funded (July to July) by a K08 from to explore lung injury in the preterm lung. This grant will be the support grant for this K08 award. The subject of this new grant is a direct extension of his research interests. He will travel to each year to perform the animal components of the experiments. He will directly supervise the lab-based work at . He also will develop new assays and reagents as needed for the project. His salary at this effort will start from this grant August 1, ( ).

, MD (0.6 calendar months) is the co-investigator responsible for the wet laboratory at . He also is responsible for lab-based work that must occur in . He will assist with assay development, study design, and data analysis. He will help supervise the technical support.

, MD, Consultant (No effort) Dr. is a world-renowned pulmonary investigator, whose lab is adjacent to our laboratory. He has been a valued collaborator and consultant for many years. One of his current interests is in airway injury and repair in transgenic mouse models. He is defining the cell lineages and gene expression networks that program epithelial injury and repair. He has shared with us reagents used to generate preliminary data for this grant and he will help us develop new reagents for use in the fetal sheep model. He also will help us with laser capture and gene expression studies.

Other Personnel

(12 calendar months) is a Research Technician III at Medical Center who is presently working on the project. She has expertise for tissue preparation, immunohistology, PCR, and the other lab-based skills for the project. Her time is necessary to assist with the extensive number of assessments.

Consortium Costs -

	YEAR 1	YEAR 2	YEAR 3	YEAR 4	YEAR 5
Direct					
Facilities and administrative					
TOTAL					

CONSORTIUM JUSTIFICATION

Consortium Arrangement - Foreign

School of Women's and Infant's Health

Consortium Costs

	YEAR 1	YEAR 2	YEAR 3	YEAR 4	YEAR 5
Direct					
Facilities and administrative					
TOTAL					

Consortium Personnel

, MD (1.2 calendar months) will be the PI for the subcontract. He is an established investigator in Maternal-Fetal medicine and Obstetrics and Gynecology. Dr. has extensive experience with the sheep model. He has worked collaboratively with Dr. . He will organize and supervise all aspects of sheep breeding, fetal surgeries, and fetal treatments. He will assist Dr. with experimental design and data analysis. No salary is requested since Dr. salary is paid by the .

(3.0 calendar months) The intensive nature of antenatal interventions and fetal treatments associated with this project require , a highly skilled fetal physiology researcher to assist us for the deliveries, fetal surgeries, and fetal treatments. In addition, Dr. will be responsible for writing and submitting animal ethics committee applications organizing breeding, scanning and transport of the ewes, and will supervise histological work including sample preparation. Dr. will supervise sample shipments and ensure that data analysis undertaken in Australia are processed and published in a timely fashion.

Research Assistant (4.8 calendar months) A research technician in in the care and maintenance of the ewes for the research program. The technician will assist with management of the ewes during the intensive experimental period, and also have responsibility for organizing supplies for the studies, and for obtaining permits and arranging the transport of samples to international collaborators at the completing of the experimental period. The research assistant will also perform cell counts, prepare slides for cytospin, and assist with studies.

OMB Number:

PHS 398 Research Plan

1. Application Type:

From SF 424 (R&R) Cover Page. The response provided on that page, regarding the type of application being submitted, is repeated for your reference, as you attach the appropriate sections of the Research Plan.

*Type of Application:

☒ New ☐ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

2. Research Plan Attachments:

Please attach applicable sections of the research plan, below.

1. Introduction to Application
(for RESUBMISSION or REVISION only)
2. Specific Aims
3. *Research Strategy
4. Inclusion Enrollment Report
5. Progress Report Publication List

Human Subjects Sections

6. Protection of Human Subjects
7. Inclusion of Women and Minorities
8. Targeted/Planned Enrollment Table
9. Inclusion of Children

Other Research Plan Sections

10. Vertebrate Animals
11. Select Agent Research
12. Multiple PD/PI Leadership Plan
13. Consortium/Contractual Arrangements
14. Letters of Support
15. Resource Sharing Plan(s)

16. Appendix

SPECIFIC AIMS.

Approximately 10% of all newborns and the majority of very low birth weight preterm infants need some assistance to breathe at birth. However the optimal maneuvers to recruit functional residual capacity (FRC) and ventilate the lungs without injury have not been defined, as stated by the . The potential to harm the lung, especially the preterm lung, is great since all resuscitation interventions use positive pressure rather than the negative intrathoracic pressure generated by spontaneous breathing (2). Initiation of ventilation with positive pressure ventilation (PPV) stretches the preterm airways, and causes airway epithelial injury which progresses to diffuse lung inflammation (3-5). . Further, there are two competing outcomes: the rapid initiation of gas exchange and the minimization of lung injury. The evaluation of lung injury following delivery room maneuvers clinically is confounded by continued PPV and oxygen exposure (4). We developed a fetal sheep model to separate injury initiated during ventilation following birth from continued ventilation (4). We can evaluate resuscitation maneuvers designed to reduce lung injury during the transition to air breathing (4, 5). Using biomarkers of airway stretch and lung inflammation developed by our group, we recently addressed an defined knowledge gap by demonstrating a benefit of PEEP and surfactant in initiation of ventilation in the preterm lung (6). Clinicians routinely introduce new treatments, such as a sustained inflation (7, 8), into newborn care without knowledge of its benefits or potential for injury. **The goal of this grant is to determine the safe and useful recruitment maneuvers for newborn resuscitation in preterm and near-term lambs, evaluations that cannot be easily assessed clinically.** Our major contributions will be to identify the mechanisms that initiate and propagate lung injury. Our recent results demonstrate that airway stretch initiates acute phase injury responses in the airways that are perhaps inevitable with the initiation of ventilation with fluid filled fetal lungs. *We hypothesize that protective ventilation strategies will minimize acute phase injury pathways in conducting airways and lung parenchyma to avoid generalized lung inflammation.* We also will test whether the severity of lung injury is dependent on gestational age (GA), and which acute phase injury pathways are activated during initiation of ventilation of very preterm, moderately preterm and term lungs. We will utilize a highly reproducible lamb model of resuscitation of the fluid filled lung and apply advanced molecular techniques to build the knowledge base for resuscitation guidelines for both preterm and term infants.

Specific Aim 1. Recruitment maneuvers at birth in the preterm. *We hypothesize that initial lung gas volume recruitment maneuvers that rapidly achieve FRC will decrease the activation of acute phase inflammatory pathways.* Initial sustained inflations are being evaluated in preterm infants because they rapidly recruit FRC (9), but little is known about the effects of these inflations on initial or subsequent lung injury. We will define the injury responses to initiation of ventilation with 1) gradual recruitment using increasing V_T, 2) rapid recruitment using sustained inflations as compared to 3) constant V_T ventilation. We also will localize activated injury pathways to the airways, lung parenchyma, and airspace fluid.

Specific Aim 2. Gestational age differences in response to recruitment maneuvers. *We hypothesize that recruitment maneuvers that are beneficial for very and moderately preterm lambs will have little benefit for the lungs of near-term lambs.* We will determine which acute phase injury pathways are activated at different stages of lung development. Although the major pathologies associated with mechanical ventilation occur in preterm infants, the majority of infants receiving PPV in the delivery room are term(10). We will test whether a ventilation strategy protective for the preterm will have benefit for near-term lamb lungs.

Specific Aim 3. Recruitment maneuvers with continued ventilation in the preterm. *We hypothesize that initiation of ventilation in newborn, preterm lambs using a lung protective strategy will decrease the propagation of the initial injury and minimize subsequent lung injury in surfactant treated preterm lambs ventilated for 4 h and 24 h.* We will test if an initial lung protective strategy can minimize the injury resulting from routine ventilation that occurs in the NICU. We will evaluate gene products and injury indicators to identify potential therapeutic targets.

These innovative studies will define the molecular and physiologic responses to recruitment maneuvers in preterm and near-term lambs, resulting in new insights into how injurious pathways progress to acute and chronic lung disease. These studies will provide a scientific basis for recommendations for clinical practices that are very difficult to verify by clinical trials.

Research Strategy

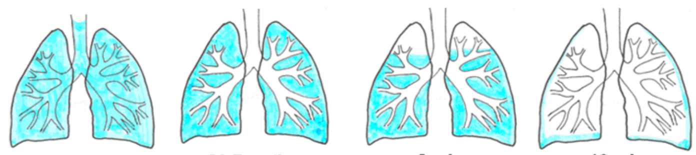
<u>Significance</u>: A rationale for this RFP for neonatal resuscitation is to develop a scientific basis for the very frequently required ventilatory assistance for preterm and term infants at birth. **I**

. These gaps are difficult to explore in the clinical setting because of the necessities to resuscitate without focusing on single components of the procedure, the great variability in the clinical status of infants, and the need to continue support beyond the specific intervention. Further we anticipate that the goal of rapidly achieving effective ventilation that is desired clinically will in general be injurious to the lungs. A clear example from Adult Respiratory Distress Trials is that High V_T improves oxygenation – a short term benefit, but is associated with increased mortality from progressive lung injury relative to lower V_T (11). The injury caused by the initiation of ventilation cannot be studied with any complexity or in depth because tissue is not available and subsequent ventilation and support can cause injury progression and obscure the intervention being tested (4). Thus, animal model experiments are essential to **fill the gaps** identified by . We have extensive experience with a preterm lamb model for fetal ventilation followed by intrauterine intervals of 15 min to 7 days to allow the injury response to develop in isolation from continued ventilation and oxygen exposure. We demonstrated that the initial injury is to the larger airways(3), that PEEP and surfactant treatments can decrease injury(6), that corticosteroids and inhibitors of IL-1β or IL-8 do not decrease injury(12, 13), and that high body temperatures can increase injury (14). We will use this model to specifically test the early response gene expression that activates inflammatory pathways and the location of expression of the inflammation within the lungs of preterm and term lambs exposed to ventilation maneuvers, which match the gaps identified by . The results will provide a deep picture of the initial injury responses of newborn lungs, which will allow us to identify less injurious strategies for the initiation of ventilation. The results also will allow us to identify potential treatment targets and biomarkers for the field. The third Specific Aim will test if a strategy to minimize injury will work in practice in the animal model by combining the resuscitation maneuver with subsequent ventilation – the clinical reality. *These results will provide information not otherwise available for future recommendation about how ventilation of preterm and term lungs can be minimally injured during resuscitation. We anticipate the results of these studies will become integrated into ILCOR recommendations to guide clinicians.*

<u>Innovation</u>: We propose translational experiments that use sheep models of lung injury that we have developed (4, 15). In this model, we will separate injury that is caused during an initial 15 min ventilation maneuver from continued ventilation, thus allowing evaluation of resuscitation maneuvers designed to reduce lung injury during the transition to air breathing at birth (4, 5). An example of the utility of this novel fetal maneuver is our recent demonstration that positive-end expiratory pressure (PEEP) decreased injury during the initiation of ventilation. We and other research groups had demonstrated a benefit of PEEP for recruitment of FRC and oxygenation, but no one had demonstrated less injury if PEEP is used (16-19). The model allows us to use specific biomarkers, developed by our group, of airway stretch (HSP70) and parenchymal injury (Egr-1) to evaluate the location of injury responses in the lungs. Many VLBW infants are severely surfactant deficient and will receive ventilation prior to surfactant treatment (20). We can evaluate the lung injury that occurs prior to surfactant treatment in surfactant-deficient animals at 118 or 128 days gestational age, prior to the variability in injury responses that occur as surfactant pools increase (13). These surfactant-deficient lambs have uniform injury responses that allow us to use small numbers of animals to acquire consistent information on injury response pathway, which is not possible in the clinical setting. By maintaining placental circulation, the fetal model also allows for return of the fetus to the uterus for prolonged periods of time (we have done up to 7 days) to measure progression of injury and evolution of molecular expression patterns (5). We will also validate protective strategies and potential therapeutic pathways in a newborn model. The sheep is an excellent large animal model for preterm human airway injury because the airways have similar cell types and cell distributions to the human airway epithelium (21-23). Similar to humans, sheep have submucosal glands in the cartilaginous airways that are thought to be the source of progenitor cells for epithelial repair (24). We have cell-type specific antibodies (See approach section) for the co-localization of inflammatory responses in epithelial cell types. We will use the recent sequencing of the sheep genome to explore in depth gene expression pathways using deep exonic mRNA sequencing, now available at . *Our proposal is innovative because it uses both a unique fetal sheep model to evaluate components of resuscitation and with* *physiology linked to advanced molecular techniques to delineate the injury pathways in a model relevant to humans.*

Background: The initiation of ventilation at birth is unique because the fetal lung must transition rapidly at birth from fluid filled airspaces to gas exchange to sustain life. Normal newborns inflate their lungs at birth by generating large negative pressure breaths, which pulls the lung fluid from the airways into the distal airspaces and parenchyma. The infant continues to clear lung fluid with subsequent inflations (25, 26). However, the majority of very low birth weight (VLBW) infants need some form of assistance at birth to breath (1, 27). The airways in the preterm lung stretch with normal ventilation pressures, and the decreased surfactant pools in the preterm lung require higher pressures for aeration and result in non-uniform expansion of the lung with areas of focal over-distension and atelectasis (Figure 1) (2, 28). The initial ventilation of the preterm lung will occur before much of the endogenous surfactant is secreted (9) and many of these infants have severe surfactant deficiency. Surfactant therapy cannot practically be given before the initiation of ventilation. In sheep models, we demonstrated that the bronchi and bronchioles were the sites of initial injury (focal expression of IL-1, Egr-1, loss of HSP 70) to a brief 15 min period of escalating V_T to 15 mL/kg (3). Acute phase response genes involved in inflammation, angiogenesis, vascular remodeling, and apoptosis were activated within the lung. Immunologically active proteins (HSP70, HSP60) were released by the airway epithelium into the airspace fluid (6). The initiation of ventilation in preterm sheep with escalating V_T for 15 min stretches the airways and causes airway epithelial injury which progresses to diffuse lung inflammation and induced lung maturation by 24 hrs (3-5). These striking effects result in part from injury of the epithelium of the small airways by movement of the air-fluid interface (3, 29). Recent clinical studies of resuscitation demonstrate that the ability of clinicians to regulate and deliver PEEP, peak pressure, and V_T to infants (or in simulation) is very poor, with the delivery of high V_T occurring frequently (30, 31). Clinicians using a T-piece resuscitation with preterm infants (< 32 weeks) provided tidal volume breaths between 0 to 31 mL/kg (31). As few as six large V_T breaths at birth can eliminate the response to surfactant treatment in preterm sheep (32). As with preterm sheep, ventilated very low birth weight (VLBW) infants have increased pro-inflammatory cytokines (IL-8, IL-1β, IL-6, and MCP-1) in tracheal aspirates soon after birth, which correlate with an increased risk of BPD (33, 34). Ventilation of preterm infants with respiratory distress increased plasma levels of IL-1β, IL-8 and TNF-α and decreased levels of the anti-inflammatory cytokine IL-10 (35). In a preliminary study of differential stretch, we escalated the V_T to 8 or 15 ml/kg by 15 min with return of the fetus to the uterus for 2 hours (Figure 2). We used 8 mL/kg because it is the normal V_T of spontaneous breathing sheep (which is higher than the 5 mL/kg V_T of healthy newborn infants). In these pilot studies, we found decreased activation of some cytokines (IL-1β, MCP-1) with the smaller V_T. Other cytokines, such as IL-6, were equally activated at both V_T of 15 mL/kg and 8 mL/kg, demonstrating a differential response between inflammatory mediators and the potential to identify pathways for intervention. Egr-1 protein expression was

Potential injurious effects of 5 mL/kg ventilation at birth



Time	Birth	20 Breaths	2 min	10 min
Location of 5ml V_T	0 mL	+5 mL to airways	5 ml to 20% of Parenchyma = **25 mL/kg**	5 ml to 100% of Parenchyma= 5 mL/kg
Outcome	Airless lung	**Overdistended airways**	**Focal overdistention**	Recommended V_T ventilation

Figure 1: The process of moving from an airless lung to an appropriately ventilated lung is likely injurious because airways are stretched. This figure gives our conception of how resuscitation can injure the lungs.

Differential lung injury with initiation of ventilation with V_T 15 mL/kg or V_T 8mL/kg

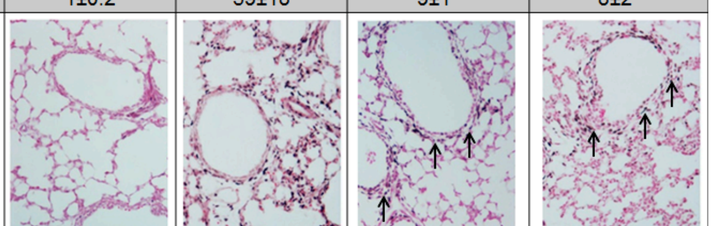
	Control	V_T 15 mL/kg	V_T 8 mL/kg	V_T 8 ml/kg + O_2
BAL Protein (mg/kg)	17±4	73±18	25±5*	20±2*
Lung Cytokine mRNA (fold increase)				
IL-1β	1±0.1	21±7	3±0.3*	2±0.3*
IL-6	1±0.2	20±6	16±2	11±4
MCP-1	1±0.2	59±18	9±1*	8±2*
Egr-1 Protein				

Figure 2: Initiation of ventilation with V_T escalating to 15 mL/kg by 15 min causes release of protein into the airspaces and inflammation (IL-1β, IL-6, MCP-1). Ventilation with a lower V_T 8 mL/kg decreased protein release and inflammatory activation. Brief exposure to 100% oxygen did not alter responses to V_T 8 mL/kg. Egr-1, an acute phase response gene, is activated throughout lung with V_T 15 mL/kg, but only around airways (arrows) with V_T 8 mL/kg. *p<0.05 vs V_T 15 mL/kg

localized to the parenchyma around the bronchioles with a V_T of 8 mL/kg, but was generalized throughout the parenchyma with 15 mL/kg ventilation (Figure 2).The decreased activation of some acute phase responses (IL-1β) with less recovery of protein in BAL by V_T 8 ml/kg relative to V_T 15 mL/kg demonstrate that the ventilator strategy can decrease injury. V_T 8mL/kg did not decrease expression of IL-6 or MCP-1, demonstrating that even low V_T resulted in injury. It is not known which of the transcription factors and inflammatory mediators that are altered by ventilation at birth will contribute to the pathways leading to BPD. Oxygen, a clinically relevant variable in resuscitation, will not be studied in this grant because preliminary data: 100% oxygen did not change acute phase responses with low V_T (Figure 2). Our in-depth studies will identify biomarkers and pathways that can be clinically useful and may be targets for new therapies.

Overview of the Experimental Plan: We propose three specific aims to answer gaps in our knowledge, as outlined by ILCOR guidelines, about the optimal V_T and recruitment maneuvers during resuscitation of both preterm and term infants. Little is known about what V_T is excessive for preterm infants and which inflammatory and developmental pathways are activated during this unique transition to air breathing. The similarity of sheep and human airway cell populations will make the results relevant for the understanding of airway injury/repair in the human. These experiments will inform clinical practice about how to think about lung injury caused at birth and ways to reduce it.

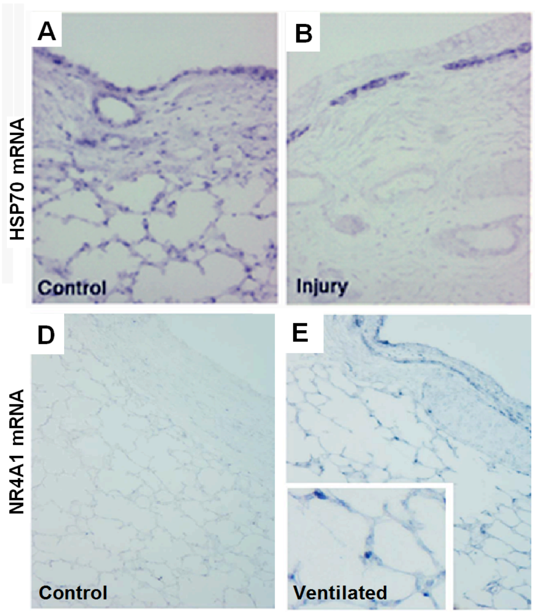
Approach: Specific Aim 1. Recruitment maneuvers at birth in the preterm.

Introduction: The transition from a fetus to a newborn requires the initiation of breathing with clearance of fluid from airways such that ventilation of the distal airspaces can occur. Spontaneously breathing term newborn rabbits quickly clear fluid from their airways at birth, with 50% of lung aeration occurring with the first three breaths(25). The rabbits increase the inspiratory volume to expiratory volume ratio to rapidly increase functional residual capacity (FRC)(36). Normal newborn infants generate very high inspiratory pressures at birth to open their lungs (37). Many preterm infants do not breathe or cannot generate enough pressure at birth and require positive pressure ventilation. Researchers have begun to explore ways to decrease injury during the recruitment of FRC. In newborn rabbits, the use of PEEP during the initiation of ventilation increased FRC (16). Surfactant treatment more uniformly distributes the FRC within the lungs (38). We recently demonstrated in the fetal lamb that PEEP and surfactant decrease lung in jury caused by a 15 min escalating V_T to 15 mL/kg (6). *Using our unique model, we will test the hypothesis that recruitment maneuvers can decrease lung injury from the initiation of ventilation at birth.*

Justification and preliminary studies:

As the concept of “open lung” ventilation became more accepted in adults with ARDS, researchers began to ask whether a sustained inflation at birth would decrease atelectasis and improve lung recruitment (39, 40). In a rat model of ARDS, a sustained inflation recruited the majority of alveoli within the first few seconds of beginning the maneuver (41). A sustained inflation for 10 to 20 seconds also increased the inspiratory volume and recruitment of FRC in rabbits at birth (42). In preterm lambs, a 1 minute sustained inflation improved oxygenation while cardiovascular variables and cerebral oxygen delivery were stable (9). Preterm infants managed with a sustained inflation of 25 cmH_2O for 15 seconds had a decreased need for and duration of mechanical ventilation (7). Spontaneously breathing very preterm infants who received short sustained inflations, verses a few manual breaths, before being placed on CPAP were less likely to need intubation in first 72 hours after birth (8). The more routine use of T-piece resuscitators has made sustained inflations more feasible in the delivery room (43). Although the physiology seems logical, very little is known about the effects of sustained inflation on lung injury or inflammatory pathways in the preterm lung.

An alternative approach to rapid recruitment of FRC with a sustained inflation would be a slow recruitment by gradual increases in V_T. Small V_T ventilation would likely cause a transient hypercarbia , which will resolve with continued ventilation. The slow increase in V_T may cause less airway distention and a more even distribution of tidal volume (Figure 1). We emphasize small airways because small airway injury continues to be prominent in children with a history of BPD (44, 45). School-age children diagnosed with moderate to severe BPD have decreased FEV_1, increased respiratory symptoms, and decreased peak flow measurements (45). Very preterm infants may be more vulnerable to airway injury since they are born with the lung at the saccular stage of development, before the formation of alveoli (46). In contrast to the adult lung, airways in the preterm lung stretch with normal ventilation and disruptions of airway epithelium are prominent in the lungs of infants who have died of RDS (28, 47). Thus the recruitment may increase FRC but has the potential to injure the airways. We reported airway injury due to the initial stretch in preterm lamb lungs based on a number of markers. (3, 6). Heat shock protein 70 (HSP70) mRNA disappears from the airway epithelium with ventilation and appears in the airway smooth muscle (Figure 3A-B). HSP70 is of particular interest because it is a chaperone protein for misfolded proteins within the cell and is an extracellular danger signal, by acting as an endogenous ligand for toll-like receptor 4 (48). HSP60 (toll-like receptor 2 ligand) also is released into the airways after ventilation (Figure 3C). Nuclear receptor 4A1 (NR4A1), an acute phase response gene involved in angiogenesis, also increases with ventilation around the airways and in the parenchyma (Figure 3D-E). Ventilation up-regulates some genes in the parenchyma (NR4A1) and down regulations others (HSP70). By using expression studies to identify multiple genes and pathways, we will be able to understand the balance of changes that translate to lung injury. A clear example of how a stretch injury causes activation of signaling pathways distinct from injury is our demonstration that high V_T ventilation induced maturational effects within 24 hr – increases in surfactant protein mRNA and Pu.1 as an indicator of lung macrophage maturation (5). Clinicians are beginning to use lung recruitment techniques for newborn resuscitation, such as a sustained inflation (7, 8), without knowledge of benefits or the potential for injury.



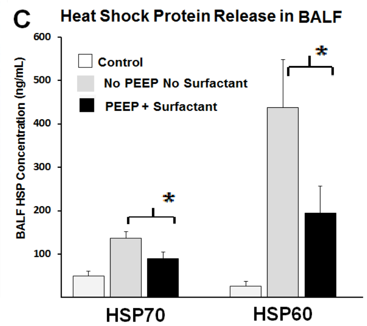


Figure 3: V_T of 15 mL/kg and fetal airways. (A-B) The ventilation depletes epithelial Heat Shock Protein 70 (HSP70) , increases HSP70 mRNA expression in smooth muscle, and decreases signal in peripheral lung compared to controls.
(C) HSP70 and HSP60 are released into BALF with ventilation and the use of PEEP and surfactant during resuscitation decrease these effects. *p<0.01
(D-E) NR4A1 mRNA increases in the smooth muscle and peripheral lung (insert) with ventilation.

Research Design: Using the fetal sheep model, we will evaluate recruitment maneuvers on lung injury during the initiation of ventilation that are now being introduced clinically (7). We will evaluate the recruitment maneuvers combined with moderate V_T ventilation of 6-8 mL/kg or large V_T ventilation of 13-15 mL/kg for 15 minutes with return of the fetus to the uterus for 30 minutes (immediate gene response profile). Based on our previous use of the fetal model, we will study: a) three 5 second sustained inflations, a time used clinically(7), and b) a 20 second inflation, a time that has been used in physiologic experiments (42).

Experiments 1a and 1b: Recruitment Injury in Preterm Lambs.

Animal Manipulation - Fetal Model: Premedicated and isoflurane anesthetized date-mated Merino Ewes are mechanically ventilated, and the fetal head and chest are exteriorized through a midline hysterectomy, while maintaining placental blood flow (4). The fetal chest is exposed to allow lung expansion that is not limited by intra-uterine pressures. The fetus is orally intubated and lung fluid is passively removed from the large airways with a syringe and suction catheter (3, 4).

The lambs will be randomized to one of four interventions: 1) ventilation with a constant V_T from birth for 15 minutes, 2) sustained inflation to 30 cmH_2O pressure for three 5 second intervals or 3) sustained inflation for 20 seconds. The lambs will then be ventilated with constant V_T for 15 minutes. 4) Gradual recruitment – lambs will be started on V_T of 2-3 ml/kg for 2 minutes, then 4-5 ml/kg for 2 minutes, then 5-6 ml/kg for 2 minutes before ventilation with a constant V_T for the remaining 9 minutes.

Recruitment Intervention	**Minutes**	***Subsequent* V_T for 15 min**	**N per group**
Constant V_T	30	6-8 mL/kg	*7*
Constant V_T	30	13-15 mL/kg	*7*
Sustained inflation - 5 s x3	30	6-8 mL/kg	*7*
Sustained inflation - 5 s x3	30	13-15 mL/kg	*7*
Sustained inflation - 20 s	30	6-8 mL/kg	*7*
Sustained inflation - 20 s	30	13-15 mL/kg	*7*
Gradual Recruitment of V_T	30	6-8 mL/kg	*7*
Surgical controls – no V_T	30	None	*6*
			54 singletons

Following the procedures the constant V_T will be 6-8 mL/kg or 13-15 mL/kg, except for the gradual recruitment procedure to 15 mL/kg. For the stretch injury, we will use Dräger Babylog 8000+ventilators for V_T ventilation based on estimated fetal weight with a ventilation rate of 40 breaths/min, a PEEP of 5 cmH_20 and an inspiratory time of 0.7 sec using heated, humidified 100% nitrogen to avoid oxidant exposure. The V_T and pressure will be monitored with a separate pneumotach, transducer, integrater, and recorder to allow us to accurately measure V_T and accumulation of FRC during maneuvers (19, 42, 49). We anticipate we may need to adjust the pressures to achieve the desired FRC volume based on experience with the maneuvers. Our goal is to achieve an FRC of about 20 mL/kg by the end of the maneuver. Control lambs will receive all the surgical procedures, and a PEEP of 5 cmH_20 but no ventilation for 15 minutes. We previously found no differences between surgical controls and unmanipulated fetal controls. In this experiment, all fetal lambs will be returned to the uterus with maintenance of placental blood supply for 30 minutes after the 15 min intervention.

<u>Experiment 1b – Effective recruitment maneuvers over time and V_T:</u> We will evaluate the most effective recruitment maneuver (determined by Experiment 1a) on initiation of ventilation with 6-8 or 13-15 mL/kg (determined in Experiment 1a) for 15 minutes with return of the fetus to the uterus for 30 minutes (immediate gene response profile, animals from 1a), 4 hours (early progressive lung injury), and 24 hours (late fully developed injury profile). Analysis of several time intervals allows us to characterize the change in molecular expression patterns as the injury progresses (5). An example of such changes in expression is HSP70 mRNA, which is: a) present in the airway epithelium of control lambs, b) lost from the epithelium 1 hour after large V_T ventilation, c) strikingly increased in epithelium 6 hours after ventilation, and d) at control levels by 24 hours.

Fetal lambs at 128±1d gestation (term~150d) will receive the same fetal procedure and ventilation as experiment 1a and be randomized to:

	Injury progression in utero					
Intervention – 15 min	**Time**	**N**	**Time**	**N**	**Time**	**N**
Constant V_T	30 min	7*	4 hr	7	24 hr	*7*
Recruitment Maneuver	30 min	7*	4 hr	7	24 hr	*7*
Surgical controls – no V_T	30 min	6*	4 hr	5	24 hr	*5*
					38 singletons	

*** Lambs from experiment 1a will be used for 30 min groups to decrease animal use**

Tissue Collection and Analysis for Experiment 1a and 1b (Box 1). Fetal lung fluid will be collected at autopsy for measurement of HSP70 and HSP60. Bronchoalveolar lavage fluid (BAL) of the left lung will be collected by repetitive saline lavage (4). BAL will be used for measurement of total protein (32), cell counts, and ELISA measurements of HSP70 and HSP60 (6). BAL will stored for further evaluations based on gene expression studies (6). Saturated PC levels will also be used to estimate surfactant pool sizes (15). After lavage, the left mainstem bronchi will be scrapped and cells collected for mRNA. Regional sampling will be done throughout the dependent and non-dependent areas of the right lung for mRNA. Tissue will be collected from the liver, thymus, posterior mediastinal lymph node for histology and snap frozen for mRNA isolation for analysis for systemic responses. The right upper lobe will be inflation fixed with 10% formalin at 30 cm H_20 and paraffin embedded (30). Samples of the trachea and right mainstem bronchi will be both formalin fixed and embedded in OTC for frozen sections for laser capture of mRNA.

Box 1: Tissue sampling and molecular applications for accessing injury	
Tissue Type	**Injury markers**
Fetal lung fluid	HSP70, HSP60
BAL (left lung)	Total protein, Cell count, HSP70, HSP60, SatPC
Trachea - Fixed	Injury scores on epithelium. HSP70, Egr-1, MCP-1, αSMA, elastin, NR4A1, CTGF Cell type markers (FoxA3, FoxJ1, p63, TTF-1)
Right Upper Lobe - Fixed	
Right Mainstem Bronchi - Fixed	
Right Lower Lobe – Frozen	*mRNA and protein analysis of*: Cytokines (IL-1β, MCP-1, IL-8, IL-6), acute phase genes (Egr-1, HSP70, Cyr61, CTGF), surfactant
Right Mainstem Bronchi - Frozen	
Left Mainstem Cell scraping	

mRNA Analysis. Total mRNA is extracted from tissues or epithelial scrapings using a Trizol method and cDNA generated (Verso cDNA kit, Thermoscientific, UK). Cells from the airway epithelial, submucosal glands, and distal lung parenchymal tissue mRNA can also be isolated from fixed blocks or frozen sections with laser capture microdissection using the Veritas Microdissection System in our Division of Pathology. Using custom Taqman gene primers (Applied Biosystems, USA) based on sheep sequences, quantitative RT-PCR will be performed with 25 ng cDNA using Taqman Master mix in 20 μl reaction on 7300 RT-PCR machine and software (Applied Biosystems, USA). 18S primers (Applied Biosystems, USA) will be used for internal loading controls, and results will be reported as fold increase over mean for control animals. For total mRNA sequencing, cDNA libraries will be prepared by the Illumina TruSeq mRNA-seq library preparation method. Clusters are generated on the flowcell by the Illumina cBot and are sequenced on the Illumina HiSeq 2000 model sequencer. The flowcell will be a SR (single-read) version 1.5 and the run was 58 cycles, 51 for

Table 1: Comparison of mRNA sequencing with RT-PCR data		
	mRNA Sequencing	**RT-PCR**
	(Fold increase over Control)	
IL-1β	11.6	16.7
IL-6	10.1	44.3
Egr-1	22.9	38.7
CTGF	12.7	8.9

the seqencing read and 7 to interrogate the indexes (barcodes) that allowed us to multiplex six samples per flowcell lane. Libraries generated are 10 to 30 million reads per sample, and analyzed on GeneSpring (Agilent Technologies, USA). We have full access to the informatics required for such analyses within the Division of Pulmonary Biology Results from mRNA sequencing will be confirmed by quantitative RT-PCR, as demonstrated by preliminary data analysis demonstrating similar fold increases for mRNA sequencing and RT-PCR using 3 lung samples from animals exposed to escalating V_T to 15 mL/kg relative to 3 unventilated controls (Table 1).

Immunohistochemistry/In situ Hybridization. Immunostaining protocols will use paraffin sections (5 µm) of formalin fixed tissues (50). Immunostaining will be visualized by Vectastain ABC Peroxidase Elite kits to detect the antigen:antibody complexes (USA). The antigen detection will be enhanced with nickel-DAB, followed by TRIS-cobalt and the nuclei counterstained with nuclear fast red or eosin (51). We currently have antibodies for many acute phase reactants (HSP70, Egr-1), cytokines (IL-1β, MCP-1) and cell specific transcription factors (FoxA1, FoxJ1, SPDEF, TTF1). Co-localization of proteins can be done using florescent antibodies and confocal microscopy (Figure 4). *In situ* localization of mRNA will be performed with digoxigenin-labeled anti-sense sheep riboprobes (Roche, USA) (3). Based on analysis of mRNA sequencing, new *in situ* probes for sheep will be used for localization. Percent staining or positive cells/hpf can be quantified on random, blinded images with Metamorph 3.5 (Universal Imaging Corp, USA).

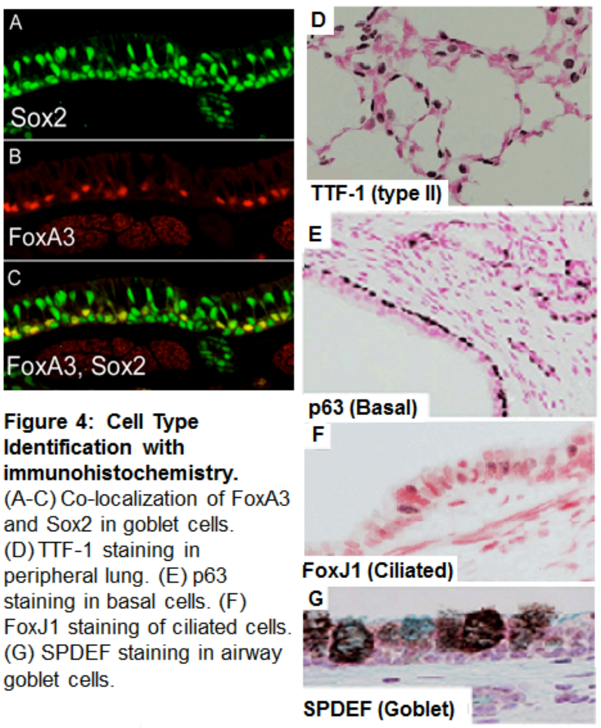


Figure 4: Cell Type Identification with immunohistochemistry. (A-C) Co-localization of FoxA3 and Sox2 in goblet cells. (D) TTF-1 staining in peripheral lung. (E) p63 staining in basal cells. (F) FoxJ1 staining of ciliated cells. (G) SPDEF staining in airway goblet cells.

Statistical Analysis. These experiments have immunohistochemical, histologic, cytokine, and protein measurements that will be compared between groups and to surgical controls. The fetal studies will have large differences between an intervention group and controls because the fetal controls are uniform and negative for inflammation. However, we are measuring changes between groups following controlled injuries. We have based our N of 7 animals per group on the examples in the table of measurements made for animals following a stretch injury of 15 mL/kg or 8 mL/kg. (Table 2) (4, 52). Six to seven animals per groups will also allow for stratification based on V_T and the recruitment maneuver. We anticipate large increases in the signals with injury and large decreases if a maneuver if effective. We will use analysis of variance with parametric and nonparametric significance tests as appropriate (4, 5).

Table 2: Power Calculations for Experiment

Variable	V_T 15 mL/kg (mean±SD)	V_T 8 mL/kg (mean±SD)	Sample Size	Power
BALF Total Protein (mg/Kg)	**75±18**	**25±5**	**4**	**90%**
IL-1β mRNA (Fold Increase vs Control)	**21±7**	**3.0±0.3**	**4**	**90%**
MCP-1 mRNA (Fold Increase vs Control)	**59±18**	**19±1**	**3**	**90%**

<u>Expected outcomes: Experiment 1a.</u> We anticipate there will be differences in the airway and peripheral gene expression responses to the recruitment maneuvers. Lung recruitment maneuvers may decrease distal lung injury, but increase airway injury. Based on our studies of PEEP and surfactant, we have adequate markers of both airway stretch and parenchymal activation to differentiate between groups and to select a recruitment maneuver for further testing (6). Analysis of complete mRNA sequencing should identify new developmental and inflammatory pathways to explore at other time points. <u>Experiment 1b.</u> We anticipate 6-8 mL/kg will have the least injury defined as less parenchyma injury and activation of acute phase response genes. There will likely be some injury to the larger airways at the lowest V_T and increased large airway injury by the higher V_T. There also will be differences between acute gene responses at 30 min and progression of injury at 4 hr and 24 hr. When possible based on availability of antibodies, mRNA changes at 30 min will be confirmed by protein changes at 4 hr and 24 hr. Analysis of changes will be made against ventilation control groups (V_T 6-8 ml/kg) and surgical controls.

Potential Problems and Alternative Strategies: We anticipate no problems performing the proposed procedures or molecular measurements based on the preliminary data and the experience we have with the model (3, 4). We will test the recruitment maneuvers at a gestation prior to the appearance of surfactant to avoid the confounding effects of differential surfactant effects (13). Our approach will be to carefully define if recruitment maneuvers can modulate injury responses using the multiple new assays that we have available. We also will selectively expand the analyses as appropriate. We will use laser capture analysis of mRNA for selected cell populations. The measurement could be expanded to additional time points or recruitment maneuvers (such as prolonged inspiratory time or variable V_T ventilation). We have assays for multiple cytokines for sheep. We have tested the antibodies for the immunolocalization and co-localization of cells and mediators and have found that most antibodies to transcription factors or intracellular signaling molecules that work in the mouse or human also work in sheep. The fetal lung fluid, BALF, and plasma can be used for proteinomic analysis with the Mass Spectroscopy Core at . This would allow us to identify additional biomarkers for early lung injury or inflammation. We have also developed a sheep primary lung epithelial cell culture system on which to test the effects of biological fluids and potential pharmacologic inhibitors. From the perspective of clinical relevance, the majority of very preterm infants are surfactant deficient to some degree. Since many preterm infants are exposed to maternal betamethasone, they may have less injury from resuscitation maneuvers (27). The study could be expanded to include maternal betamethasone. We previously showed that maternal betamethasone decreased lung and airway injury from an escalating V_T injury and subsequent ventilation in preterm newborn sheep (12).

Approach: Specific Aim 2. Gestational age differences in response to recruitment maneuvers.

Introduction: Mechanical ventilation is the primary clinical variable that drives bronchopulmonary dysplasia (BPD) (53, 54). The anatomy of BPD (fewer alveoli, decreased microvasculature, and airway remodeling) can be caused by mechanical ventilation in ventilated preterm baboons and sheep (55, 56). BPD occurs in 25% of VLBW infants and is almost a uniform outcome for infants born with birth weight below 750g (53, 57). Strategies to reduce mechanical ventilation at birth tend to decrease BPD (58, 59). However poorly controlled interventions to decrease oxygen exposure and mechanical ventilation have not consistently decreased the incidence of BPD in very preterm infants (59, 60). Although the risk of BPD increases as gestational age decreases (53), BPD does occur in more mature preterm infants and occasionally in term infants exposed to prolonged mechanical ventilation and oxygen. Of note, even moderately preterm infants without a history of lung disease as infants can have pulmonary function tests indicative of airway disease at term and in childhood (44). *We will test the hypothesis that recruitment maneuvers will increase lung injury in very preterm lambs and have little effect on lung injury in the near-term lamb.*

Justification and feasibility: The fetal human lung is in the saccular stage of lung development between 23 and 32 weeks of gestation (61) and epithelial injury during this period of development likely disrupts normal developmental pathways. The cell types present in the tracheal and airways change and mature across gestational ages. In rhesus monkeys, the airway epithelium has ciliated cells and mucous secretory cells in the pseudoglandular and cannicular stages, with basal cells and small mucous granular cells appearing by the late cannicular phase (62). All cell types are present in the primate airways by the saccular phase of development. Sheep develop alveoli earlier in gestation than do humans (63). At 100d gestational age, the fetal sheep lung is in the late cannicular phase of lung development, similar to a 22 week gestational human. By 120d the fetal sheep lung has transitioned to the late secular/early alveolar phase of lung development, similar to human airways at about 30 weeks. By 140d of gestation,

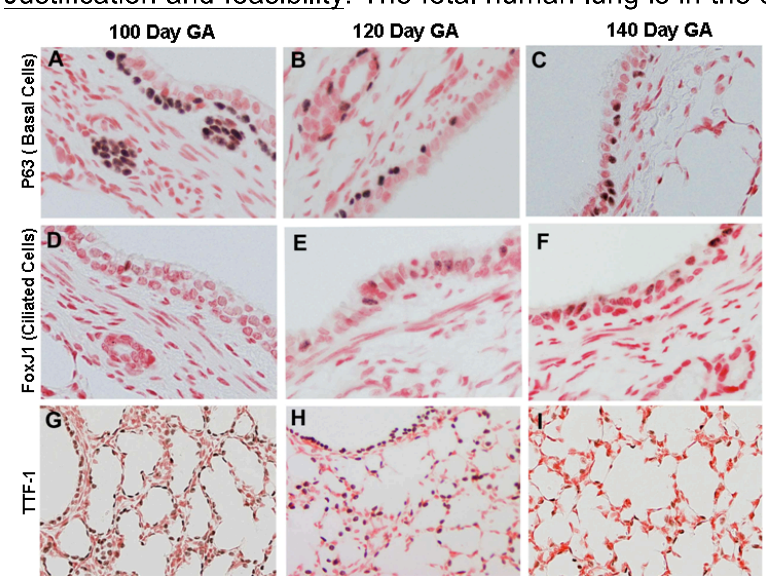


Figure 5: The anatomy of fetal sheep changes from GA 100d to 140d. Using cell-type specific markers, we co-localize injury pathways and specific cell populations across gestational ages. Basal cells (p63 staining), Ciliated cells (FoxJ1) and TTF-1 positive cells all change in location and intensity across GA in the distal parenchyma.

the fetal sheep has well defined alveolar structures and that are similar to a human infant after term birth. At 110d GA, fetal lambs ventilated in utero for 1 hour then recovered for 12 hours had epithelial disruption, increased proliferation (Ki67 staining), decreased secondary alveolar crests, and alveolar wall thickening (64). Fetal lambs at 110d GA that were ventilated in utero for 12 hours then returned to uterus for 7 days had a thickened epithelium, increased smooth muscle actin and collagen, and more simplified airspaces compared to control lambs (65). Newborn rats are born with saccular lungs. When ventilated with high V_T, the lungs have different gene expression patterns than adult rats, demonstrating an effect of developmental stage on inflammatory activation (66). Figures 2-4 show lung tissue from sheep at about 129d GA. Although all the cell types are present in epithelium of the fetal lung by the third trimester in fetal sheep, the distribution of cell types and structure of lung parenchyma strikingly changes between 100 days and 140 d GA (Figure 5). Initiation of ventilation during these different developmental stages should activate different injury and developmental pathways which may contribute to the risk of BPD at early gestation ages.

Fluid moving across epithelial cells generates high surface forces that distort the cells (29). Similarly, epithelial cells that are stretched in vitro have striking acute phase injury responses (67), and the preterm sheep trachea is more distendable with pressure than is the term trachea (28). The injury should be more severe in more immature airways with more deformation. As the infant gets closer to term, the degree of injury should decrease and the injury pathways that are activated may differ. We found minimal injury from ventilation of near term lambs (140 days gestational age (GA), term ~ 150 days) with V_T of 8 mL/kg suggesting airway stretch is of less concern in term infants (14, 68). At 133 days GA, newborn lambs have variable lung injury responses that decrease with the endogenous surfactant pool size increases (lambs with >5% of term surfactant levels have less inflammation)(13). No studies have evaluated injury responses in the preterm or term lung across relevant periods of lung development.

Research design: Fetal lambs at 118d, 128d, and 140d gestational age are randomized to either 1) 15 min of constant V_T ventilation or 2) a beneficial recruitment maneuver (developed in Aim 1a,b) followed by 15 min of ventilation. The lambs are then returned to the uterus for 30 minutes (total experimental period - 45 minutes) to allow us to assess acute phase responses. The 140d GA (near-term) lambs will be more mature and more difficult to injure. We will compare a normal V_T of 6-8 mL/kg with a high V_T of 25-30 mL/kg to achieve an injury. Although these V_T may seem excessive, clinicians asked to resuscitate newborn lambs based on the ILCOR guidelines gave an average V_T of 17.8 mL/kg (69), and clinicians resuscitating preterm infants (< 32 weeks) provided tidal volume breaths between 0 to 31 mL/kg (31).

Intervention – 15 min ventilation			**Time for injury progression**	
GA	Initial ventilation	Followed by 15 min V_T (mL/kg)	**In utero min**	**N**
118d	Constant V_T	6 to 8	30	*7*
118d	Recruitment Maneuver	6 to 8	30	*7*
128d	Constant V_T	6 to 8	30	*7**
128d	Recruitment Maneuver	6 to 8	30	*7**
140d	Constant V_T	6 to 8	30	*7*
140d	Recruitment Maneuver	6 to 8	30	*7*
140d	Constant V_T	25 to 30	30	*7*
140d	Recruitment Maneuver	25 to 30	30	*7*
118d	Surgical Control	No V_T	30	*5*
128d	Surgical Control	No V_T	30	*5**
140d	Surgical Control	No V_T	30	*5*
			52 singletons	

*** Lambs from experiment 1a will be used to decrease animals use**

Expected Outcomes: We will collect tissue to perform molecular measurements similar to those described for Specific Aim 1 (Box1). We expect that although the recruitment maneuver may be beneficial in the 128d GA lambs, it may cause more damage to the 118d epithelium from increased stretch and sheer effects. Although increased V_T will be used with the term animals, there will likely be less injury, irrespective of the recruitment maneuver. We anticipate that the injury will increase in the very preterm lambs (118d) and different patterns of gene activation will occur between the groups. This will provide clinicians and researchers with additional biomarkers for lung injury that can be evaluated in clinical trials.

Potential Problems and Alternative Strategies: Our goal for this study of stretch mediated injury at birth and its moderation by a recruitment maneuver will require us to perform a few preliminary studies to identify the V_T at 118d and 140d GA that will initiate injury and the pressures needed to recruit FRC. Multiple reports demonstrate that V_T and not pressure is the variable causing injury(70). We anticipate higher peak inspiratory pressure may be required for the very preterm lungs to achieve our target volumes than for the 128d or near-term lambs. However, higher V_T will be needed to injure the near-term lungs. We have the experience working with fetal sheep across this range of gestation and do not anticipate problems achieving our study goals. We do not know how much difference there will be in acute phase response or in location of injury in the airways or parenchyma. This study focuses only on the very early response gene activation that occurs within 30 min. If we identify differences that could be of clinical interest, we can expand the studies to longer intervals in utero after the 15 min of ventilation to capture progression of injury.

Approach: Specific Aim 3. Recruitment maneuvers with continued ventilation in the preterm.

Introduction: The studies in Aims 1 and 2 allow us to explore lung injury and progression in the absence of continued ventilation. However, the majority of newborn preterm infants that require ventilation at birth also receive assisted ventilation after stabilization at delivery. The additional ventilation may amplify any injury response or negate a lung protective strategy used for the initial resuscitation (4). Ventilation studies with newborn lambs incorporate the physiologic changes that occur normally at birth, specifically the decrease in pulmonary vascular resistance with shunting across the ductus arteriosus, and the loss of the low pressure placental circulation. **We hypothesize that initiation of ventilation in newborn, preterm lambs using a lung protective strategy (developed in Aim 1) will decrease the initial propagation of the initial injury (30 min) and the subsequent progression of lung injury in surfactant treated preterm lambs ventilated for 4 hrs and 24 hrs.** We have previously shown that CPAP from birth can decrease lung injury relative to gentle ventilation in more mature lambs (71). We do not propose a CPAP study because lambs at 128d GA will not breath spontaneously at birth (15). Investigators are just beginning to determine some of the molecular or inflammatory pathways that lead to BPD (6). We will assess whether resuscitation maneuvers can modulate inflammatory pathways and acute phase responses in newborn lambs. *Our goal is to test if lung protective strategies at resuscitation will benefit the lungs with continued ventilation during neonatal transition.*

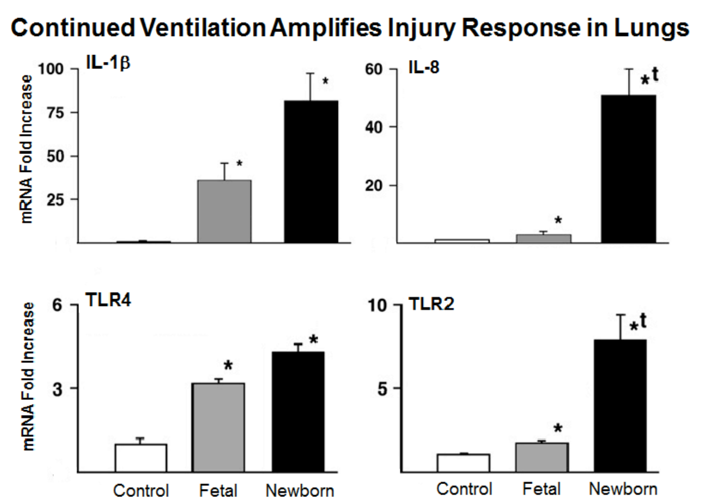


Figure 6: Cytokine and Toll-like Receptor mRNA increases with ventilation.
* p<0.05 vs Controls, t p<0.05 vs Fetal. (Hillman NH *et al*. AJRCCM 2007)

Justification and feasibility: We compared initiation of ventilation with high V_T in fetal lambs maintained on placental support with newborn lambs with the same initial ventilation followed by surfactant treatment and ventilator support for 3 hours (Figure 6) (4). We found a large amplification of pro-inflammatory cytokines with continued ventilation. Along with the inflammatory response, high V_T ventilation also increased toll-like receptor (TLR) mRNA in the lungs (Figure 6). TLR4, TLR2, and Serum Amyloid A3 mRNA also increased in the liver (4). The location of cytokine mRNA expression patterns differed between groups (Figure 7) (3). MCP-1 expression increased in the mesenchyme surrounding the bronchioles (Figure 7B) with fetal ventilation and throughout the lung parenchyma with three hours of ventilation after the initial V_T injury (Figure 7C). IL-6 mRNA increased minimally 3 h after the fetal V_T injury, but was strikingly amplified throughout the lung with continued ventilation of the newborn (Figure 7E-G) Cytokine expression in the lung is quantified relative to unventilated controls in Figure 7 (D,H). We used this tissue for mRNA sequencing as preliminary data for this grant to test if the technique would

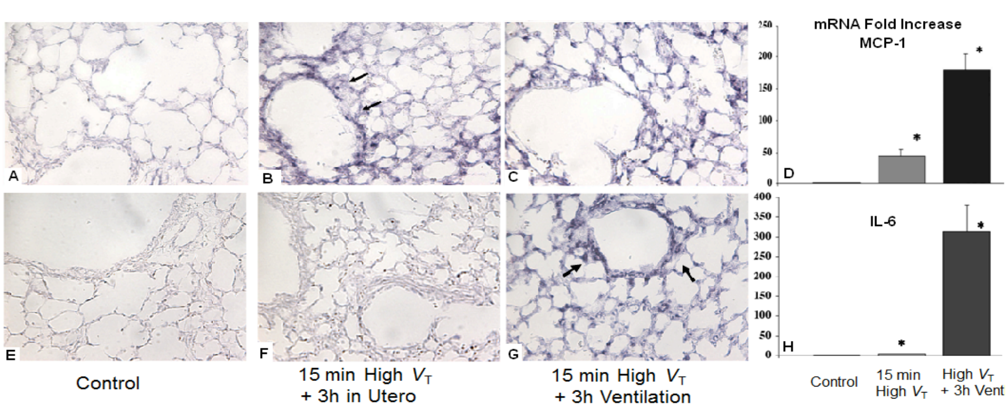


Figure 7: Differential Cytokine profiles for MCP-1 and IL-6. *In situ* localization of MCP-1 and IL-6 mRNA within the lung. (D,H) Quantification on mRNA as fold increase over control.
* p<0.05 vs Controls (Hillman NH *et al*. Ped. Research 2010)

give us similar results and could identify new pathways of interest in the preterm sheep lung (Table 2). Relative to controls, we found similar amplification of injury for IL-1β, IL-6, and TLR2. We also identified that , the core intracellular signaling protein for TLR pathways, is increased with 15 min of stretch injury and further increased with continued ventilation. In contrast, ICAM-1 (an adhesion molecule important in inflammation) and MMP2 (a metalloproteinases involved in extracellular matrix degradation) increased with stretch injury, but were strikingly suppressed with ventilation compared to controls. These preliminary measurements were made using 3 tissue samples per group. These are examples of how mRNA sequencing will allow us to identify new genes and pathways that participate in stretch injury of the preterm lung. For the first time we will begin to understand the balance of changes that translate to lung injury. A clear example of how a stretch injury causes activation of signaling pathways distinct from injury is our demonstration that 15 min of high V_T ventilation induced lung maturation within 24 hr as indicated by increases in surfactant protein mRNA and Pu.1 as an indicator of lung macrophage maturation (5). This aim will combine the animal model with advanced molecular techniques to determine if recruitment maneuvers designed to decrease lung injury during resuscitation are sustained with ventilation of the newborn.

Table 2: mRNA fold changes in Fetal and Newborn models

	Fetal	Newborn
	(Fold Change vs Control)	
IL-1β	11.6	46.6
IL-6	10.8	288
TLR2	1.5	8.8
MYD88	1.5	3.6
ICAM-1	3.8	-6.6
MMP2	2.4	-4.5

Research design: Experiment 3 - Recruitment maneuvers and ventilation of newborn preterm lambs.

Newborn lambs at 128±1d gestation will be randomized to constant V_T ventilation or a recruitment maneuver identified in Aim 1 and then constant V_T ventilation for 15 minutes. The lambs then are surfactant treated and ventilated for 30 minutes, 4 hours or 24 hours, each interval selected to optimize the evaluation of injury progression and for comparison with fetal animals from Aim 1b.

Initial intervention – 15 minutes	**Surf TX**	**Subsequent Ventilation**	**Animal # (128d GA)**
Standard ventilation (V_T 7-8 mL/kg)	+	30 min	*7*
Protective ventilation (Aim 1)	+	30 min	*7*
Standard ventilation (V_T 7-8 mL/kg)	+	4 h	*7*
Protective ventilation (Aim 1)	+	4 h	*7*
Standard ventilation (V_T 7-8 mL/kg)	+	24 h	*7*
Protective ventilation (Aim 1)	+	24 h	*7*
Unventilated Controls	None	None	*5*
		47 singletons	

Animal Manipulation. Date-mated 128 d preterm lambs will be operatively delivered, a tracheostomy performed, and lung fluid removed and the lamb delivered. Prior to initiation of ventilation, the newborn lambs will be randomized either: 1) 15 min of a standard ventilation (V_T 8 ml/kg) *or* 2) a lung protective maneuver (determined by Aim 1), such as a sustained inflation of 30 cmH_2O for 20 seconds prior to V_T 8 ml/kg for 15 min. Ventilation will be continued (rate 40 breaths/min, inspiratory time 0.7 s, FiO_2 0.40) with a Drager BL8000+ ventilator (Drager, Lubeck, Germany) using a time-cycled, volume-guarantee mode and 8 L/min flow with heated and humidified gas and PEEP 5 cmH_2O. V_T will be escalated to achieve the target V_T of 7 to 8 mL/kg by 5 minutes. At end of the initial 15 minute initial ventilation, lambs will be treated with 100 mg/kg porcine surfactant (Curosurf®, Chiesi Pharmaceuticals, Italy), as we have done previously(12).

Following the initial 15 min ventilation period, the volume guarantee ventilation mode will be set at 7 mL/kg and the lambs ventilated for 30 min, 4 hrs or 24 hrs with a heated and humidified 40% oxygen and air mixture (50 breaths/min, PEEP 5 cmH_2O, inspiratory time 0.7 s). An umbilical artery catheter will be used for blood gas sampling. Blood gases and ventilator setting will be monitored at 5, 10, 15, and 30 min and then every 30 minutes to 4 h, then hourly during the 24 h ventilation. Lung compliance will also be monitored. Umbilical vein catheters will be placed for continuous infusion of Remifentanil (0.05 µg/kg/h; Ultiva, Glaxo Smith Kline,) and Propofol (0.1 mg/kg/h; Repose, Norbrook Laboratories, ,). The $PaCO_2$ will be targeted at 50 mmHg by adjusting V_T. FiO_2 will be adjusted to target a PaO_2 of 50 to 100 mmHg, with continuous pulse oximetry monitoring and PaO_2 measurements. Temperature is maintained under radiant warmers with core temperatures continuously monitored. The lambs will have plasma samples and tracheal aspirates collected (Box 2). The lambs will be killed with a lethal intravenous dose of pentobarbital (100 mg/kg, Valabarb, Jurox, NSW,) 45 minutes, 4h or 24 h after delivery. In addition to the lung tissue sampling described in Aim 1 (Box 1) and tissue from other organ will be collected (liver, thymus, posterior mediastinal lymph node, spleen).

Box 2: Plasma and Tracheal Aspirate sampling

Group	15m	45m	2h	4h	8h then q4h
45 min	X	X			
4 h	X	X	X	X	
24 h	X	X	X	X	X

Expected Outcomes: We will perform similar measurements as for Specific Aim 1. We anticipate that the recruitment maneuver will decrease the acute phase activation and release of inflammatory substances into the BAL at 45 minutes. There will be decreased pro-inflammatory responses with less inflammatory cell recovery into the BAL at 4 hours with the protective strategy. We will compare the magnitude of changes between the ventilated newborn lambs with those evaluated in the fetal model at corresponding time points (Aim 1). We anticipate that continued ventilation, even in the setting of surfactant treatment, will amplify the injury response and may blunt repair pathways that may begin after the injury in the fetal model. Although we currently have measures for determining differences between groups (Box 1), the information derived from mRNA sequencing in Specific aims 1 and 2 will be used to identify additional predictors and pathways. The ventilation for 4 and 24 hours will allow us to test the utility of the recruitment maneuvers in a real life simulation. If the recruitment maneuver decreases the release of immune active substances, then by 24 hours we should have better ventilation and oxygenation for the recruitment maneuver group relative to the constant V_T "standard care" group. The collection of plasma and tracheal aspirates throughout the experiments will allow us to identify inflammatory and protective substances that are accessible clinically for evaluation .

<u>Potential Problems and Alternative Strategies</u>: We anticipate no problems performing the proposed measurements. We have extensive experience with the ventilation of newborn lambs in the facilities in . As with the fetal surgery model, the newborn model can be expanded to test a variety of resuscitation maneuvers. Novel pathways that are discovered can be explored in future studies using alternative resuscitation techniques and inhibitors to target particularly injurious pathways that would be given within 24 hr of the birth and ventilation. A limitation is the 24 hr interval of ventilation relative to identifying structural changes that would reflect progression to BPD.

Work Plan for the Specific Aims. These animal studies will be performed in . The advantages of performing the animal procedures and tissue collections in are 1) the sheep are healthy, selected for uniformity of age and size, and are genetically uniform Merino ewes and 2) the breeding is performed by experienced personnel, (Over the past 10 years, the singleton pregnant ewes are at precisely the gestational ages that we have requested); 3) the ewes are kept in large outside paddocks and given supplemental food to optimize nutrition and moved at the same time to the research facility; and 4) all studies will be performed at the peak of the breeding season. Animals of this quality and uniformity are not available in the US. About 40 to 50 fetal surgeries and deliveries per year will be performed. The sum of the animals for the experiments described is 191, but there will be some losses and a need for a few pilot studies to develop the FRC recruitment maneuvers. Autopsies will be performed by Drs. and tissues will be shipped to for molecular analysis.

VERTEBRATE ANIMALS

The vertebrate animals to be used in this study are the sheep. The sheep studies will be performed at the . Detailed protocols and standard operating procedures are in place to ensure appropriate handling, minimize pain, and suffering to the sheep. The protocols are fully reviewed by the Animal Ethics Committee for the . All animal work is supervised by the veterinary staff at the . These experiments are performed at the Large Animal Facilities on the

1. Detailed description of the proposed use of the animals. This translational research project will use preterm fetal sheep as a model for neonatal lung injury and repair because the injury procedures can be carefully designed and accurately controlled and lungs can be removed for multiple assessments of injury and repair. The fetus will be exposed to the injuries while exteriorized from anesthetized ewes. Fetal sheep will be returned to uterus while maintaining placental blood flow and maternal anesthetics. Fetal lambs will be delivered at intervals up to 24hr after an intervention and euthanized. Intravascular arterial and venous catheters may be used for fetal evaluation and drug administration. For study of ventilated newborns, fetal sheep are delivered of anesthetized ewes, intubated, and fully sedated following placement of umbilical artery and venous lines. The lambs are ventilated and cared for using modern neonatal ICU equipment for up to 24hr.

2. Justification. This translational research will evaluate fetal and newborn lung injury from resuscitation maneuvers. Lung injury assessments require not only the lung, but also a normal systemic circulation and systemic injury response systems. We have chosen sheep for these studies because we can use the same equipment used clinically to achieve injury, measure physiologic outcomes, and collect multiple biochemical and molecular measurements on the same animal. Results with preterm sheep were predictive of surfactant treatment and metabolism, and the sheep is the standard large animal model in general use in perinatal medicine. It is impractical to evaluate clinically relevant lung injuries using fetal rodents. The fetal model will

allow us to maintain the pregnancy to study epithelial and airway injury. While fetal baboons could be used, they are far more valuable and not appropriate for a mechanistic project of this scope. Animals are minimized using a group size of about 6 under standardized conditions in a very uniformly responding animal model.

3. Veterinary care. The animals will be bred for the project with supervision by the veterinary services of the . The ewes are under the supervision of the veterinary services from the for the duration of the experimental period.

4. Procedures to minimize pain and discomfort. For deliveries the ewes are pre-anesthetized with Ketamine (10 mg/kg) and medetomidine given by IV injection. The ewes are then given spinal-epidural anesthesia (4mL of 2% lidocaine). For fetal surgeries, the ewes are given the same pre-anesthetics, intubated and anesthetized using isoflourane and mechanical ventilation. The inhalant anesthetic will provide anesthesia for the lamb during fetal surgery. The ewes will be recovered after surgery, receive infiltration of the incision site with ropivacaine and monitored until walking and feeding. For post-operative analgesia, a fentanyl patch will be applied to the groin. For preterm lambs to be ventilated after operative delivery, an umbilical vein catheter is placed at delivery and the animal is anesthetized with continuous infusions of Remifentanil (0.5 μg/kg/hr) and Propofol (0.1 mg/kg/hr). We have developed detailed standard operating procedure with the help of a veterinary sheep anesthetist, , DVM, and these analgesia conform to the veterinary guidelines.

5. Euthanasia. The ewes and fetal sheep will be euthanized by lethal injection of 100 mg/kg pentobarbital administered in the umbilical vein of fetus and peripheral vein for the ewe. The animal is then exanguinated. This method of euthanasia is recommended for sheep by the

REFERENCES

1.

2.

3.

4.

5.

6.

7.

8.

9.

10.

11.

12.

13.

14.

15.

16.

17.

18.

19.

20.

21.

22.

23.

24.

25.

26.

27.

28.

29.

30.

31.

32.

33.

34.

35.

36.

37.

38.

39.

40.
41.

42.

43.

44.

45.

46.
47.

48.

49.

50.

51.

52.

53.

54.

55.

56.

57.

58.

59.

60.

61.

62.

63.

64.

65.

66.

67.

68.

69.

70.

71.

Consortium/Contractual arrangements: The project will be performed as a collaborative project between and The The principal investigator and the co-investigators on this grant have had a long-standing collaboration over 21 years. There have been about 25 collaborative publications in the last 6 years.

Programmatically, Dr. is the Principal Investigator, while Dr. is the principal investigator of the sub-contract award and Dr. is a co-investigator. Dr. will be the overall director of the scientific component of the grant. Drs. will supervise all aspects of sheep breeding and fetal surgeries. The sheep will be purchased by from professional sheep breeders. For the duration of the experiments, the sheep will be housed at the facilities of .

The grant will be administered by the Business Office of the Division of Neonatology under the auspices of the Sponsored Programs Office of the . The administrative business director will correspond with the Business Office of the School of Women's and Infant's Health, as required. The consortium costs will be paid for by as a quarterly payment to the . Approximately 30% of the direct costs of the grant will be paid as a sub-contract to the

The grantee will be because the primary scientific input in to the formulation of the grant idea, generation of Preliminary data and the experimental approach, data analysis for the grant is generated/performed in . The collaboration represents a true complementation of the expertise in such that the combined output is more than the sum of the parts.

RESOURCE SHARING

1. Data Sharing – Does not apply since requested funds are below $500,000 in any year.
2. Model organisms – Development of model organisms is not planned in the grant.

We have fully shared probes and reagents with other investigators over the years and will continue to do so.

PHS 398 Checklist

OMB Number: 0925-0001

1. Application Type:

From SF 424 (R&R) Cover Page. The responses provided on the R&R cover page are repeated here for your reference, as you answer the questions that are specific to the PHS398.

* Type of Application:

☒ New ☐ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

Federal Identifier:

2. Change of Investigator / Change of Institution Questions

☐ Change of principal investigator / program director

Name of former principal investigator / program director:

Prefix:
* First Name:
Middle Name:
* Last Name:
Suffix:

☐ Change of Grantee Institution

* Name of former institution:

3. Inventions and Patents (For renewal applications only)

* Inventions and Patents: Yes ☐ No ☐

If the answer is "Yes" then please answer the following:

* Previously Reported: Yes ☐ No ☐

4. * Program Income

Is program income anticipated during the periods for which the grant support is requested?

☐ Yes ☒ No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period	*Anticipated Amount ($)	*Source(s)

5. * Disclosure Permission Statement

If this application does not result in an award, is the Government permitted to disclose the title of your proposed project, and the name, address, telephone number and e-mail address of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information (e.g., possible collaborations, investment)?

☒ Yes ☐ No